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Comparison of Human Dental Pulp and Bone Marrow Stromal Stem Cells by cDNA Microarray Analysis

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We compared the gene expression profiles of human dental pulp stem cells (DPSCs) and bone marrow stromal stem cells (BMSSCs) as representative populations of odontoprogenitor and osteoprogenitor cells, respectively. Total RNA from primary cultures was reverse-transcribed to generate cDNA probes and then hybridized with the Research Genetics human gene microarray filter GF211. The microarrays were analyzed using the PATHWAYS software package. Human DPSCs and BMSSCs were found to have a similar level of gene expression for more than 4000 known human genes. A few differentially expressed genes, including collagen type XVIII α1, insulin-like growth factor-2 (IGF-2), discordin domain tyrosine kinase 2, NAD(P)H menadione oxidoreductase, homolog 2 of *Drosophila* large disk, and cyclin-dependent kinase 6 were highly expressed in DPSCs, whereas insulin-like growth factor binding protein-7 (IGFBP-7), and collagen type I α2 were more highly expressed in BMSSCs. Furthermore, we confirmed the differential expression of these genes by semiquantitative polymerase chain reaction (PCR) and northern blot hybridization. The protein expression patterns for both IGF-2 and IGFBP-7 correlated with the differential mRNA levels seen between DPSCs and BMSSCs. This report describes the gene expression patterns of two distinct precursor populations associated with mineralized tissue, and provides a basis for further characterization of the functional roles for many of these genes in the development of dentin and bone. (Bone 29:532–539; 2001)

Key Words: Dental pulp stem cells (DPSCs); Bone marrow stromal stem cells (BMSSCs); cDNA microarray; Osteoblast; Odontoblast.

Introduction

Different stem cell populations have been identified with the ability to regenerate all or part of the tissues from which they were originally derived.[24] We recently identified a population of human dental pulp stem cells (DPSCs) with the ability to develop into odontoblasts, cells that form the mineralized matrix of dentin.[25] In vivo, human adult teeth do not undergo turnover and exhibit a limited capacity to repair the dental-pulp structure in contrast to the regenerative capacity of marrow and bone. Therefore, the functional significance of adult pulp tissue harboring a DPSC population remains unclear. However, these findings are in accord with previous reports describing stem cell populations from other nonregenerating tissues, such as brain and skeletal muscle.[13,43,63,66] Although the exact origin and location of DPSCs within dental pulp has yet to be determined, their ability to form a dentin/pulp-like complex in vivo is akin to the bone-forming capacity of bone marrow stromal stem cells (BMSSCs), "mesenchymal stem cells," following transplantation into immunocompromised animals.[25,29,38] In addition to the formation of ectopic, lamellar bone, BMSSC transplants are often associated with a vascularized marrow organ containing hematopoietic and adipogenic elements, features never observed in the corresponding DPSC transplants.

In spite of the fact that odontogenic and osteogenic progenitors are quite distinct in their ontogeny[51] and developmental potentials in vivo,[25] both populations demonstrate a remarkable similarity with respect to their expression of various proteins commonly present in the extracellular matrix of bone and dentin.[25] One exception is the odontoblast-specific gene, dentin sialophosphoprotein (DSPP), which encodes two proteins (dentin sialoprotein and dentin phosphoprotein), both of which have been associated with early dentinogenesis and are absent in bone.[22,40] Functionally active odontoblasts and osteoblasts are thought to arise from their respective precursor cell populations through a series of transitional stages based on morphological, biochemical, and molecular criteria.[7,32,50,55] During development, osteogenesis is initiated by a cascade of signals driven by master regulatory genes such as Cbfa1,[19] a transcription factor that is also thought to play a pivotal role in early tooth development.[11,65] However, little is known about the precise molecular cues responsible for the differentiation of putative precursor cell populations leading to the eventual formation of dentin or bone. To further characterize human DPSCs and BMSSCs, we elected to analyze their gene expression profiles by employing high-density cDNA microarray technology. We also assessed the effectiveness of using cDNA microarray technology to compare patterns of gene expression between these two stem cell populations.

The development of cDNA microarrays in the various forms (nylon filters, glass slides, and more recently oligonucleotide silicon chips), has emerged as a more rapid and efficient alternative to other gene discovery techniques such as serial analysis of gene expression (SAGE), differential display, and expressed sequence tag (EST) sequencing.[4,16,59] Microarray technology has made it easier to analyze simultaneously the expression of thousands of genes between multiple samples to study cell

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Table 1. Polymerase chain reaction (PCR) primers list

Gene	Primer sequence	Accession no. (product size)
Insulin-like factor binding protein-7 (IGFBP-7)	Sense: 5′-tgatatgcatgcttttcttctg-3′; antisense: 5′-tggttgatgcccttacatga-3′;	S75725 (394 bp)
Collagen type I α2 (COLLIα2)	Sense: 5′-aggccctcaaggtttccaagg-3; antisense: 5′-ccagaccattgtgtcccctaa-3′	Y00724 (233 bp)
Collagen type XVIII α1 (COLL18α1)	Sense: 5′-gtgcagtatcatgccctgtg-3′; antisense: 5′-aacaggtctgggttttgtgc-3′	AF018081 (156 bp)
Insulin-like growth factor-2 (IGF-2)	Sense: 5′-ctctccgtgctgttctctcc-3′; antisense: 5′-cgggccagatgttgtacttt-3′	S77035 (196 bp)
Cyclin-dependent kinase 6 (CDK6)	Sense: 5′-tgtttcagcttctccgaggt-3′; antisense: 5′-tggaaactatagatgcgggc-3′	XM_004987 (142 bp)
Homolog 2 of *Drosophila* large disk (DLG2)	Sense: 5′-taagtggggagctccagaga-3′; antisense: 5′-tctgacgtagagggagcgtt-3′	XM_006009 (257 bp)
NAD(P)H menadione oxidoreductase (NMOR1)	Sense: 5′-ggctgaacaaaagaagctgg-3′; antisense: 5′-cggaagggtcctttgtcata-3′	J03934 (159 bp)
Discordin domain tyrosine kinase 2 (DDR2)	Sense: 5′-caagaacagccctattccca-3′; antisense: 5′-caggcactgacagcatcact-3′	X74764 (331 bp)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Sense: 5′-agccgcatcttcttttgcgtc-3′; antisense: 5′-tcatatttggcaggtttttct-3′	M33197 (816 bp)

development, signal transduction, apoptosis, disease diagnosis and characterization, therapy response, drug discovery, and drug safety evaluation.[5,14] In this study, we used the Research Genetics microarray filter GF211 system to generate a gene expression profile of DPSCs and BMSSCs based on over 4000 known human genes. The gene profiles from four individual DPSC and BMSSC samples were compared. Differentially expressed genes between human DPSCs and BMSSCs were confirmed by semiquantitative polymerase chain reaction (PCR) and northern blot hybridization.

Materials and Methods

Subjects and Cell Culture

Normal human impacted third molars were collected from two adult men (22 and 31 years old) and two women (19 and 27 years old) at the Dental Clinic of the National Institute of Dental and Craniofacial Research under approved guidelines set by the NIH. The extracted molars were placed into regular growth medium and the pulp tissue extracted and prepared as single cell suspensions as previously described.[25] Bone marrow mononuclear cells (BMMNCs), processed from marrow aspirates of normal human adult volunteers (two women 19 and 32 years old, and two men 21 and 35 years of age), were purchased from Poietic Technologies (Gaithersburg, MD) and washed in growth medium. Primary cultures were generated and maintained as previously described.[25]

cDNA Microarray Filter Analysis

GF211 Human "Named Genes" GeneFilters Microarrays Release 1 (Research Genetics, Huntsville, AL) nylon membranes, containing over 4000 known human genes, were used for hybridization studies. The filters were prehybridized with 5.0 μg of denatured cot-1 DNA (Life Technologies), 5.0 μg poly-dA (Research Genetics), and 5.0 mL MicroHyb (Research Genetics) hybridization solution for 2 h at 42°C. Total cellular RNA from primary DPSC and BMSSC cultures was prepared from single cell suspensions, using the RNA Stat-60 extraction method (Tel-Test, Inc., Friendswood TX), according to the manufacturer's recommendations. Probes were generated from reverse-transcribed total RNA (1.0 μg) incubated with 2.0 μg oligo-dT (Research Genetics), 6.0 μL 5× first-strand buffer (Life Technologies), 1.0 μL dithiothreitol (DTT; Life Technologies), 1.5 μL reverse transcriptase (Superscript II, Life Technologies), 1.5 μL dNTP mixture containing dATP, dGTP, dTTP at 20 mmol/L (Pharmacia), and 10 μL ^{33}P-dCTP (3000 Ci/mmol, ICN Radiochemicals) for 90 min at 37°C. Purified, labeled probes were added to the prehybridization solution and left to hybridize with the GF211 membranes for 18 h at 42°C. Washes were done twice at 50°C in 2× SSC with 1% sodium dodecylsulfate (SDS) for 20 min and once at room temperature in 0.5× SSC with 1% SDS for 15 min. The membranes were aligned on a PhosphorImaging screen and exposed for 16 h at room temperature. The screens were scanned using a PhosphorImager and saved as digital images using IMAGEQUANT (Molecular Dynamics, Inc., Sunnyvale, CA). The Research Genetics software PATHWAYS was used for data analysis. Differential gene analysis was performed for all pairwise comparisons (n = 16) between the four DPSC and BMSSC samples using a threshold of twofold or greater in hybridization intensity.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

First-strand cDNA synthesis was performed on total RNA from primary DPSC and BMSSC cultures using a first-strand cDNA synthesis kit (Gibco BRL, Life Technologies) with an oligo-dT primer. First-strand mixtures were used for PCR as described previously[25] using 10 pmol/L of each human specific primer set (**Table 1**). The reactions were incubated in a PCR Express Hybaid thermal cycler (Hybaid, Franklin, MA) at 94°C for 2 min, for 1 cycle, then 94°C (45 sec), 56°C (45 sec), 72°C (60 sec) for either 20, 25, 30, or 35 cycles. Following PCR amplification, samples were analyzed by 1.5% agarose gel electrophoresis, by ethidium bromide staining. Band intensities were compared with the corresponding GAPDH controls using IMAGEQUANT software.

Northern Blot Analysis

Poly-(A) mRNA was then prepared from total RNA extractions using the Ambion mRNA isolation kit, according to the manufacturer's specifications, from primary DPSC and BMSSC cul-

tures. Following electrophoresis, poly-(A) (2 μg per lane) was transferred to a nylon membrane (Zeta-Probe) and prehybridized in QuickHyb hybridization solution (Stratagene, Cedar Creek, TX) at 68°C for 15 min. Probes were generated from purified PCR products using the primer sequences listed in Table 1, by random labeling with (γ-^{32}P) dCTP (Dupont New England Nucleotide) using the Stratagene Prime It II labeling kit, then hybridized with the filters at 68°C for 1 h. Washes were performed twice in 2× SSC, 0.1% (w/v) SDS for 15 min at room temperature, followed by one wash in 0.1% SSC and 0.1% (w/v) SDS at 68°C for 30 min. The membranes were exposed to a PhosphoImager cassette for 16–72 h with a Cronex intensifying screen (Du Pont, Wilmington, DE). Band intensities relative to GAPDH was assessed by PhosphorImager analysis using IMAGEQUANT software.

Western Blot Analysis

Lyophilized conditioned media prepared from DPSC and BMSSC cultures grown in 2% serum were reconstituted in loading buffer under reducing conditions. Equivalent amounts of diluted protein samples were separated on a 4%–20% Tris-glycine SDS-polyacrylamid-gel electrophoresis (PAGE) gel (Novex, San Diego, CA). The proteins were then transferred onto BA-S 85 nitrocellulose membranes (Schleicher & Schuell, Keene, NH) and blocked for 3 h at room temperature in 3% (w/v) bovine serum albumin (BSA) and 3% normal goat serum. Mouse anti-human insulin-like growth factor binding protein-7 monoclonal antibody (Diagnostic Systems Laboratories, Inc., Webster, TX) was added directly to the blocking solution (1/1000 dilution) for 1 h at room temperature. Filters were washed and then incubated with a 1:50,000 dilution of goat anti-mouse immunoglobulin G (IgG; H+L) conjugated to horseradish peroxidase (HRP; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for 1 h at room temperature. Following immunolabeling, the membranes were washed and reacted with Super Signal chemiluminescence HRP substrate (Pierce Chemical Co., Rockford, IL) according to the manufacturer's recommendations, then analyzed using Kodak X-Omat film (Kodak, Rochester, NY).

Enzyme-linked Immunosorbent Assay

Control media containing 2% fetal calf serum (FCS) and conditioned media from confluent first-passaged cultures of BMSSCs and DPSCs were filtered through a low-protein-binding 0.22 μm filter. All samples were subjected to acid treatment, then used to measure IGF-2 concentration by enzyme-linked immunosorbent assay (ELISA) using an IGF-2-specific ELISA kit (DSL-10-9100; Diagnostic Systems Laboratories) according to the manufacturer's recommendations.

Flow-cytometric Analysis of Cell-cycle Antigens Ki-67 and PCNA

Cell-cycle phases were determined by flow-cytometric analysis based on expression of the Ki-67 antigen (G1, G2/M, S) and the S-phase-specific proliferating cell nuclear antigen (PCNA) as previously described.[39] Trypsin/ethylene-diamine tetraacetic acid (EDTA) digested single cell suspensions (2×10^5 cells) were fixed with cold ethanol (70%) on ice then incubated with the monoclonal antibodies specific to the cell-cycle-associated antigens Ki-67 conjugated to FITC and PCNA conjugated to PE (1/10) (Dako Corp., Carpinteria, CA). Replicate tubes were incubated with FITC- and PE-conjugated isotype-matched control antibodies of irrelevant specificity (Becton Dickinson) and analyzed using a FACScalibur flow cytometer (Becton Dickinson). Positive expression was defined as the level of fluorescence >99% of the corresponding isotype-matched control antibodies.

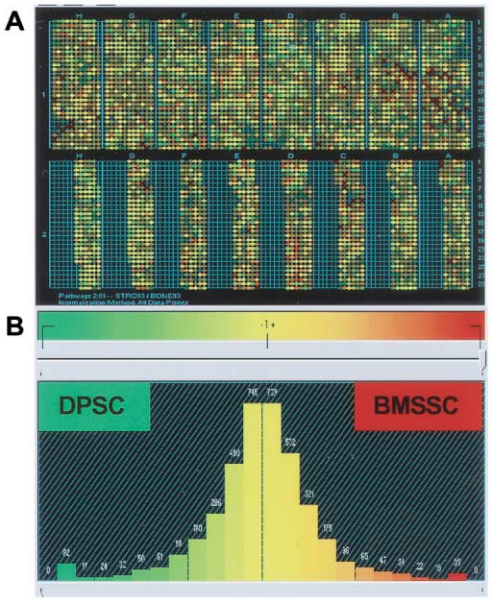

Figure 1. Gene expression profile comparison. PATHWAYS software-derived picture graph depicting hybridization intensity ratios based on color designations for DPSC (green) and BMSSC (red) expressed cDNA spots on the GF211 microarray filter (A). Intermediate colors (yellow-orange) represent commonly expressed genes. A representative histogram is shown demonstrating the frequency of commonly expressed genes on the GF211 filter (B).

Results

DPSCs and BMSSCs Share Similar Gene Expression Profiles

We utilized cDNA microarray technology to compare multiple gene expression profiles representative of normal human adult DPSCs (n = 4 individuals) and BMSSCs (n = 4 individuals). Labeled cDNA probes were generated from total RNA extracts isolated from primary cultures and then hybridized with the Research Genetics GF211 filter. In total, four filters were probed twice, alternating between different BMSSC and DPSC samples. The filters were scanned on a Molecular Dynamics PhosphorImager to generate a digital image using IMAGEQUANT software. The images were imported and analyzed using the PATHWAYS software. Gene intensity values were normalized to the control genes on each filter. Direct comparisons were made between all DPSC and BMSSC expression profiles based on a color-coded designation (BMSSC, red; DPSC, green) for each hybridized dot (**Figure 1**A), where intermediate colors (orange and yellow) represented mutually expressed genes of similar intensities. Pairwise comparisons between all BMSSC and DPSC samples demonstrated a similar expression profile of approximately 4400 known genes present on the GF211 microarray (Figure 1B). Several genes coding for extracellular matrix components (collagen types III and V, osteopontin, osteonectin, matrix gla-protein, decorin, biglycan, and alkaline phosphatase), cell adhesion molecules (VCAM-1, CD44, and integrins β_1; α_2; α_v, and β_3), growth factors (IGF-1, PDGF, FGF-2, TGF-β1, BMP-2, BMP-4, and BMP-7), and regulators of transcription (Cbfa1, Msx1, Msx2, c-FOS, c-Jun, and Jun-B) have previously been

Table 2. Genes differentially expressed in human BMSSCs and DPSCs

Gene name (accession no.)	Mean ratio ± SE (n = 16)
Highly expressed in BMSSCs	
Collagen type I, α-2 (AA490172)	7.94 ± 0.24
Insulin-like growth factor binding protein-7 (T53298)	8.58 ± 0.85
Highly expressed in DPSCs	
Collagen type XVIII, α1 (N81029)	2.86 ± 0.07
Insulin-like growth factor-2 (N74623)	3.96 ± 0.40
Discoidin domain tyrosine kinase 2 (AA243749)	3.17 ± 0.11
NAD(P)H:menadione oxidoreductase (AA458634)	2.31 ± 0.12
Homolog 2 of *Drosophila* large disk (R60019)	2.25 ± 0.24
Cyclin-dependent kinase 6 (H73724)	2.57 ± 0.30

KEY: BMSSCs, bone marrow stromal stem cells; DPSCs, dental pulp stem cells.

associated with the development and function of osteoblasts and/or odontoblasts.

Confirmation of Differentially Expressed Genes

Pairwise cross-comparative analysis of all the individual samples (n = 8) between the two subject groups failed to find any genes that were expressed exclusively by either BMSSCs or DPSCs using the commercially available filters. However, differences in hybridization intensities were observed for a proportion of the mutually expressed genes (mean ± SE: 14 ± 2.6%, n = 16), based on a threshold of twofold or greater difference in signal intensity. All differentially expressed genes identified between DPSC and BMSSC samples were visually examined to eliminate the possibility of "bleed over," which is the phenomenon that causes false-positive signals from an adjacent, highly expressed gene. Following exclusion of those genes that were not differentially expressed in all DPSC/BMSSC comparisons (n = 16), only a few genes proved to be differentially expressed on a consistent basis between all DPSC and BMSSC samples tested (**Table 2**). Due to the high sensitivity of microarray analysis to detect transcripts of low abundance, differentially expressed genes were confirmed using either northern blot hybridization (**Figure 2**A,B) or semiquantitative RT-PCR (Figure 2C). Collagen type XVIII α1 (COLL 18 α1), insulin-like growth factor-2 (IGF-2), discordin domain tyrosine kinase 2 (DDR2), NAD(P)H menadione oxidoreductase (NMOR1), homolog 2 of *Drosophila* large disk (DLG2), and cyclin-dependent kinase 6 (CDK6) were all found to be highly expressed in DPSCs. Insulin-like growth factor binding protein-7 (IGFBP-7), and collagen type I α2 (COLL 1 α2) were observed to be more highly expressed in BMSSCs when normalized to GAPDH expression (**Table 2**).

Higher Proliferation Rate of DPSCs Correlates to Higher Expression of IGF-2 and Decreased Levels of IGFBP-7

Subconfluent cultures of DPSCs consistently displayed a higher incidence (96%) of cells in the DNA synthesis phase of the cell cycle when compared with BMSSCs (68%), based on flow-cytometric analysis of the cell-cycle-specific antigens PCNA and KI-67 (**Figure 3**). Due to differences in cell proliferation rates, we elected to confirm the protein expression of IGF-2, a known stimulator of cell growth, together with its low-affinity binding protein, IGFBP-7, previously shown to act as an inhibitor of cell proliferation. Because both IGF-2 and IGFBP-7 are secreted as soluble factors, we measured protein levels in conditioned media taken from DPSC and BMSSC cultures following acid extraction. Conditioned media from DPSC cultures contained significantly higher levels of IGF-2 (103 ng/mL) when compared with conditioned medium from the corresponding BMSSC cultures (22 ng/ml) using an IGF-2-specific ELISA kit (**Figure 4**). Conversely, BMSSC cultures produced higher levels of IGFBP-7 protein than DPSC cultures, as assessed by western blot analysis (Figure 4). Therefore, the differential protein expression observed for IGF-2 and IGFBP-7 correlated with the discordant mRNA expression seen for both proteins using cDNA microarray analysis.

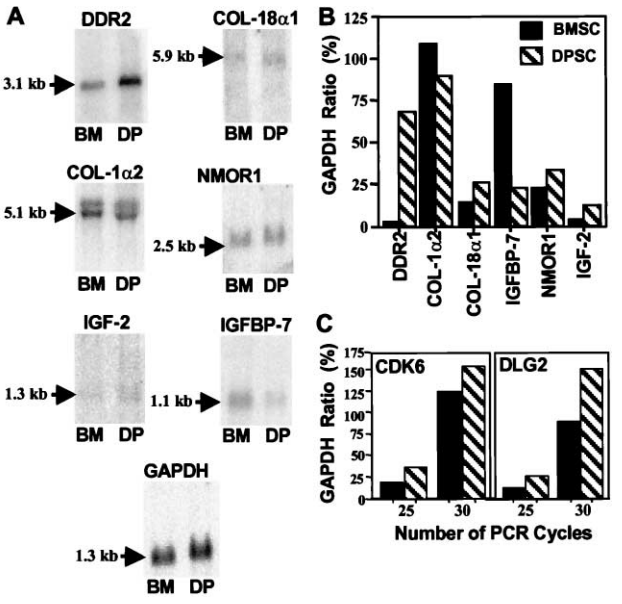

Figure 2. Confirmation of differentially expressed genes. (A) Representative northern blot hybridizations of mRNA derived from DPSCs (DP) and BMSSCs (BM) with ^{32}P-labeled probes for insulin-like growth factor binding protein-7 (IGFBP-7), collagen type 1 α2 (COLL 1α2), collagen type XVIII alpha 1 (COLL 18 α1), insulin-like growth factor-2 (IGF-2), discordin domain tyrosine kinase 2 (DDR2), NAD(P)H menadione oxidoreductase (NMOR1), and GAPDH. (B) Gene expression levels are shown as a ratio of GAPDH expression. (C) Representative gene expression levels of homolog 2 of *Drosophila* large disk (DLG2) and cyclin-dependent kinase 6 (CDK6) by semiquantitative RT-PCR. Band intensities of ethidium bromide-stained gels were quantified as a ratio of GAPDH expression at the corresponding amplification cycle.

Discussion

In the present study, we compared representative gene expression profiles between several DPSC and BMSSC samples, using the Research Genetics GF211 cDNA microarray filter system. This technology offers the advantage of comparing multiple profiles generated from different subjects using the same filter (two or three hybridizations) or identical filters from the same batch. During the analysis, pairwise comparisons were performed between all DPSC and BMSSC samples to minimize the variation in gene expression that may arise through differences in gender and age. The efficacy of using high-density cDNA filters for gene expression profiling has previously been demonstrated in studies characterizing different human cancers and analyzing tumor progression.[61,70]

Comparisons between DPSCs and BMSSCs showed a similar gene expression profile for many genes represented on the GF211 filter, such as those encoding for collagen types III and V, noncollagenous extracellular matrix components (osteopontin,

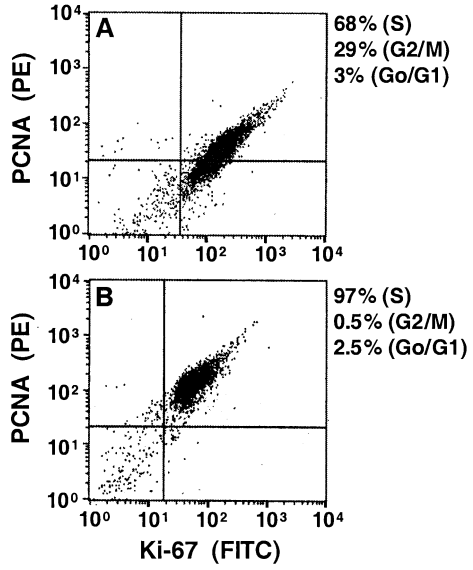

Figure 3. Analysis of the cell-cycling status of DPSCs and BMSSCs. Dual-color immunofluorescence flow cytometric analysis of single cell suspensions of BMSSCs (A) and DPSCs cells (B), immunoreacted with the PCNA-specific monoclonal antibody coupled to PE (y axis) and the Ki-67-specific antibody coupled to FITC (x axis). The dot plot histogram represents 2×10^4 events collected as listmode data. The vertical and horizontal lines were set to the reactivity levels of $< 1\%$ mean fluorescence obtained with the isotype-matched control antibodies, IgG1/(FITC) and (PE) (Becton Dickinson). The average percentage of cells in the different cell-cycle phases of G0/G1 ($PCNA^-/Ki\text{-}67^{-/+}$), G2/M ($PCNA^-/Ki\text{-}67^{++}$) and S ($PCNA^+/Ki\text{-}67^{+/++}$) are compiled from two BMSSC and DPSC samples.

osteonectin, matrix gla-protein, decorin, biglycan, and alkaline phosphatase) and several cell adhesion molecules (VCAM-1, CD44, integrins β_1, α_{v2}, α_v, and β_3). Functionally, these molecules have been associated with the initiation of mineralization and with bone homeostasis,[3,12,26,47,54,64,73] in addition to influencing the process of dentinogenesis.[6,8] Other commonly expressed genes included those coding for various growth factors

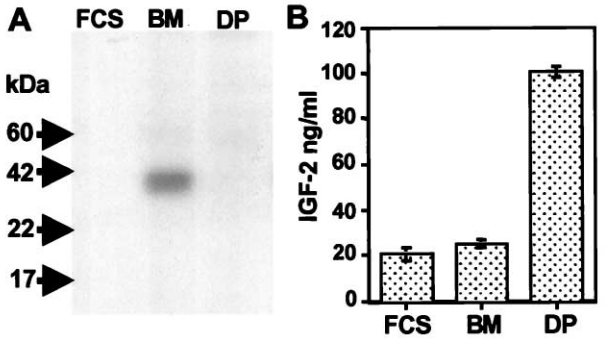

Figure 4. Expression of IGFBP-7 and IGF-2 protein. Representative western blot analysis of control media containing 2% serum (FCS) and conditioned media derived from DPSC (DP) and BMSSC (BM) cultures immunoreacted with a IGFBP-7-specific monoclonal antibody (A) as described in *Materials and Methods*. Control media containing 2% FCS and aliquots of conditioned media were also assessed for the presence of IGF-2 protein (B), following acid extraction using an IGF-2-specific ELISA kit. The average IGF-2 concentration was taken from two separate DPSC and BMSSC cultures.

(IGF-1, PDGF, FGF-2, TGF-β1, BMP-2, BMP-4, and BMP-7) implicated as strong promoters of osteogenesis and the formation of mineralized bone matrix.[18,32,42,45] Some of these factors are also involved in tooth morphogenesis,[30,42,56] by orchestrating the activation of early response molecules such as sonic hedgehog, which in turn regulates other cytokines and transcriptional regulators.[53,65]

One critical transcription factor, Cbfa1, was first described as an osteoblast-specific regulatory molecule that interacts with the promoters of several bone-associated markers including bone sialoprotein, osteocalcin, alkaline phosphatase, and collagen type I.[19] Cbfa1 null mice exhibit a complete absence of bone formation but also demonstrate an arrest in tooth development at the cap/bell stage.[37,65] In addition, mutations of the Cbfa1 gene in both humans and mice manifest as cleidocranial dysplasia, a syndrome associated with various skeletal and tooth abnormalities.[46,52] Therefore, Cbfa1 expression seems to be a critical factor for the development of both normal bone and teeth. Human DPSCs and BMSSCs were found to express low levels for two isotypes of Cbfa1, Cbfa1 type I (Pebp2αA/Cbfa1), and type II (Osf2/Cbfa1 or til-1). In some cases, both Pebp2αA and Osf2, were found to be expressed only marginally above the background intensity. It is yet to be determined whether the majority of DPSCs and BMSSCs constitutively express low levels of Cbfa1, or whether Cbfa1 is expressed by only a subpopulation of cells within primary cultures.

The homeobox transcription factor, Msx2, and a number of other molecules (c-FOS, c-Jun, and Jun-B) that comprise the AP-1 complex transcription factor, were also expressed at low levels by both DPSCs and BMSSCs. These genes are regulated by TGF-β superfamily members and fibroblast growth factors and are important inducers of early bone and tooth development.[9,36,65] Some of these genes may act in part by regulating the expression of master control genes that direct the processes of osteogenesis and odontogenesis. For example, Msx2-deficient mice demonstrate delayed growth and ossification of the skull and long bones correlating with a downregulation of Cbfa1 expression.[17] Interestingly, another homeobox gene family member, Msx1, was expressed at high levels by both DPSCs and BMSSCs. Msx1 is an important transcription factor regulating inductive signaling during tooth morphogenesis.[10,65] Both Msx1-deficient mice and human Msx1 mutations exhibit cleft palate and abnormalities of craniofacial bone and altered tooth development.[58,67] Collectively, these studies imply that the molecular pathways involved in tooth development may be similar to those that influence the formation of bone. This is consistent with the findings of the present study demonstrating that DPSCs and BMSSCs express similar gene expression profiles in vitro, even though DPSCs and BMSSCs are isolated from different anatomical sites derived from distinct origins during embryogenesis. This point was illustrated by the fidelity of DPSCs and BMSSCs to regenerate the distinct microenvironments from which they were originally derived in xenogeneic transplants.[25]

Few differentially expressed genes were consistently expressed between all DPSC and BMSSC samples tested, using a threshold of twofold or greater difference in hybridization intensity. Moreover, we failed to detect any genes that were exclusively expressed by either cell population. Functionally, the DPSCs demonstrated a higher proliferation rate over BMSSCs in vitro, correlating with increased levels of the cell-cycle-specific kinase, cdk6, and the mitogen, IGF-2. Cyclin-dependent protein kinase 6 is activated by D-type cyclins, ultimately driven via growth factor stimulation to promote the progression of cells from G1 to the start of DNA synthesis.[21,27] Murine cell lines overexpressing human IGF-2 demonstrate increasing levels of cyclin D1, and shortening of cell-cycle time.[72] Similarly, over-

expression of IGF-2 in the human colon carcinoma cell line, Caco-2, not only increases proliferation but also prevents cell-cycle arrest in G2-M, which is necessary for the expression of differentiation markers for this cell population.[71] In transgenic mice, the overexpression of IGF-2 results in a variety of symptoms associated with Beckwith–Wiedemann and Simpson–Golabi–Behnmel syndromes, including skeletal abnormalities and cleft palate.[20,62] The relevance of the higher levels of IGF-2 observed for DPSCs requires further investigation. It has been speculated that high levels of IGF-2 found in dentin (up to 9- and 30-fold greater than IGF-1 and TGF-β, respectively) may be involved in maintaining the dental pulp microenvironment by preventing resorption of mineralized dentin under steady-state conditions.[23]

Both insulin-like growth factors and receptors (IGF-RI and IGF-RII) have also been implicated as regulators of bone turnover,[44] a system complicated by the presence of several binding proteins subdivided into high-affinity IGFBPs (1–6) and low-affinity IGFBPs (7–10).[35,60] In the present study, higher levels of IGFBP-7 mRNA and protein were detected in BMSSCs when compared with DPSCs. IGFBP-7 is a 37 kDa protein also known as Mac25/prostacyclin-stimulating factor/IGFBP-related protein-1. This protein is localized in a range of normal human tissues, such as heart, brain, lung, kidney, liver, gut, skeletal muscle, skin, and blood vessels, and has decreased expression in different cancer cell lines.[15,49] IGFBP-7 has previously been shown to suppress the growth rate of a p53-negative osteosarcoma cell line, following transfection with constructs containing either the IGFBP-7 or p53 cDNA.[34] This IGFBP-7-mediated suppression of cell growth was also observed for human cervical carcinoma cells (HeLa), and murine embryonic carcinoma cells (P19).[33] The increased levels of IGFBP-7 in BMSSCs may also contribute to the lower proliferation rate seen in vitro.

Clearly, a substantial amount of work is required to fully explore the functional role of all the differentially expressed genes identified using microarray analysis. Although beyond the scope of the present study, future work will focus on the functional significance of various genes of interest such as IGF-2, IGFBP-7, and others such as collagen type XVIII (COLL18α1), a basement membrane heparan sulfate proteoglycan localized at the perivascular basal laminae.[28] This molecule is prevalent in the liver and has been identified in other tissues such as kidney and lung. Together with collagen type XV, COLL18α1 belongs to a new collagen subfamily, named the multiplexins.[57] High expression of COLL18α1 by DPSCs may help to provide vascular homeostasis within the pulp microenvironment. Recently, studies have identified the C-terminal fragment of COLL18α1 as endostatin, an antiangiogenic peptide with strong tumor-suppressing activity.[2,48]

Notably, several other genes, with tumor-suppressor properties, were also highly expressed by DPSCs when compared with BMSSCs. The gene encoding for homolog 2 of *Drosophila* large disk (DLG2) is a member of the large disk gene family, and is a homolog to the *Drosophila* tumor suppressor dlg-A gene that mediates signal transduction through protein-protein interactions at the cytoplasmic surface of the cell membrane.[41] Another signaling molecule, discoidin domain receptor tyrosine kinase 2 (DDR2), has been identified in connective tissues and functions as a mediator of cross talk between extracellular matrix proteins and cytoplasmic effectors.[68,69] Studies of invasive tumors have shown that these cells express high levels of another discoidin domain family member, DDR1, whereas transcripts for DDR2 express only in the surrounding stromal cells.[1] In addition, DPSCs expressed higher levels of the flavoprotein, NAD(P)H: menadione oxidoreductase (NMOR1), which acts to catalyze reduction of various redox dyes and quinones. This enzyme is thought to provide a protective effect against tumors caused by the actions of quinones and their metabolic precursors.[31] These intriguing observations beg the question as to whether increased expression of COLL18α1, DLG2, DDR2, and NMOR1 by DPSCs conveys some type of defensive advantage against cancer formation in dental pulp, given the rarity of tumors classified as originating from human adult dental pulp tissue.

In summary, we have described an application for cDNA microarray technology for characterization and comparison of the molecular fingerprints of odontogenic and osteogenic stem cell populations. The gene expression profile data demonstrated an accurate representation of many proteins previously shown to be expressed by both DPSCs and BMSSCs.[25] Moreover, our studies demonstrated a correlation between the expression of mRNA and protein for two differentially expressed genes, IGF-2 and IGFBP-7. Therefore, cDNA microarray technology may be used to further study gene expression patterns of DPSCs and BMSSCs at various transitional stages during cell differentiation, offering the unique opportunity to identify important molecular pathways involved in the developmental progression of DPSCs and BMSSCs toward mature odontoblasts and osteoblasts, respectively.

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